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Liposomes and tear fluid.
I. Release of vesicle-entrapped carboxyfluorescein

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The successful application of liposomes as a topical ophthalmic drug delivery device requires knowledge of vesicle stability in the presence of tear fluid. The release of 5-carboxyfluorescein from large unilamellar liposomes in the presence of rabbit tear fluid was studied in vitro as a function of bilayer cholesterol content. Reverse evaporation vesicles were prepared from egg phosphatidylcholine, stearylamine and varying amounts of cholesterol. Both the rate and the extent of fluorescent dye release were significantly increased in the presence of rabbit tear fluid at all cholesterol levels. However, by incorporating increasing amounts of cholesterol in the vesicle bilayers, tear-induced leakage was reduced. The release kinetics reported in this study are similar to those observed in the presence of human serum. While serum-induced leakage is attributed to high-density lipoprotein-mediated destabilization, reported differences in tear protein composition suggest some other, as yet unidentified, factor.

Introduction

Liposomes, as potential carriers of drugs and biologicals, have stimulated considerable interest as delivery devices both in vivo and in vitro. One of the major obstacles encountered in the application of liposome technology for in vivo drug delivery via the circulatory route has been the removal or destruction of the liposome before any benefit can be achieved. Liposomes injected into the circulatory system are generally taken up rapidly by cells of the reticuloendothelial system [1] and, even before phagocytosis, serum components act to disrupt liposome integrity. This results in the premature loss of associated agents [2,3]. Resistance to in vivo degradation has been variously accomplished through the inclusion of cholesterol [4–6], saturated phospholipids [3], sphingolipids [3,4], and glycolipids [7].

Even though liposomal drug delivery within the body has received considerable attention, successes have been limited. While much of the reason for this lack of success stems from the problems outlined above, the impenetrability of most of the vascular endothelium generally prohibits extravascular movement of liposomes injected into the blood-stream [8]. For these reasons, effective liposome-mediated delivery has been most successful when the preparations are delivered directly to the anatomical site where an effect is desired, e.g., synovial joints [9], the skin [10], and the eye [11].

Our interest has been focussed on the use of liposome entrapment to improve delivery of biological and pharmaceutical agents to ocular tissues following topical application. Conventional eye

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drops cannot provide sustained therapeutic drug levels in the precorneal area. Drug delivery by this method is pulsed with an initial high drug level followed by dilution and removal by normal lacrimation. While some success has been achieved utilizing liposomes as carriers for ocular drug delivery [12–14], only one preliminary investigation appears to have been undertaken to examine the effect of tear fluid on liposome stability [15]. In this communication we demonstrate that vesicle permeability to the water-soluble dye, carboxyfluorescein, is increased in the presence of tear fluid and that release can be restricted by the incorporation of cholesterol in the liposomal bilayers.

Materials and Methods

Animals and tear collection. The animals used were normal female New Zealand white rabbits, 12–14 weeks old and 2.0–2.5 kg in weight. Tears were collected from the lower eye-lid margins of rabbits with 10 μl micropipettes. Extreme care was taken to avoid irritating the eye so as not to stimulate tearing.

Chemicals. Egg phosphatidylcholine, cholesterol, stearylamine, Hepes and octyl β-D-glucopyranoside were obtained from Sigma (St. Louis, MO). 5-Carboxyfluorescein was purchased from Calbiochem-Behring (La Jolla, CA) and purity was confirmed by HPLC. All other chemicals were reagent grade.

Preparation of liposomes. Liposomes containing the aqueous dye, carboxyfluorescein, were prepared using the reverse evaporation method [16]. A total of 66 μmol of lipid, dissolved in organic solvent, was dried onto the sides of a pear-shaped flask using a stream of nitrogen. Residual solvent was removed in vacuo for a minimum of 30 min. The dried lipid was redissolved in 3 ml of diethyl ether to which 1 ml of 200 mM carboxyfluorescein in 100 mM Hepes (pH 7.4) was added. A stable emulsion was created by sonication at 100 W for 3 min using a probe sonicator (Braunsonic 1510). The sample was kept on ice and was purged with nitrogen throughout the procedure. The ether was then removed by rotary evaporation under reduced pressure in a 30°C waterbath and the resulting vesicles were then either used immediately or stored under nitrogen at 5°C.

Prior to use, the liposomes were washed twice by ultracentrifugation in 100 mM Hepes (pH 7.4) at 118000 × *g* for 20 min at 5°C. The final pellet was gently resuspended with 0.5 ml of buffer and then passed through a Sephadex G-50 (coarse) column (3.0 × 25 cm) pre-equilibrated with Hepes buffer. The liposomes were collected in the void volume and used immediately.

Carboxyfluorescein-release assay. Dye efflux was monitored essentially as described by Weinstein et al. [17]. Washed liposomes were mixed with an equal volume of either rabbit tears or Hepes buffer and incubated at 37°C. At specified times, 20-μl aliquots were diluted 5-fold in buffer. A 30-μl aliquot was then mixed with 2 ml of buffer in a cuvette for fluorescence measurements. Fluorescence, before and after the addition of 100 μl of Triton x-100 (10%, v/v), was measured using a Perkin-Elmer 650-10M fluorescence spectrophotometer. All measurements were made at room temperature using excitation and emission wavelengths of 488 and 520 nm, respectively, with slit widths of 5 nm.

For each sample, the percent remaining carboxyfluorescein (latency) was calculated by using the fluorescence reading obtained after detergent lysis as 100% efflux. The half-times for carboxyfluorescein leakage were calculated according to Allen and Cleland [2] using the initial linear portions of the plots of log[carboxyfluorescein latency] versus time.

Freeze-fracture electron microscopy. Liposome samples were quenched from room temperature by plunging them into partially solidified Freon 22. No cryoprotectant was used. The samples were freeze-fractured using a Balzers freeze-fracture unit at −100°C. Fractured samples were platinum shadowed at a 45° angle. The carbon replicas were cleaned in concentrated bleach for about 30 min and then in chloroform/methanol (2 : 1, v/v) for a further 10–15 min.

Vesicle sizing. Quasi-elastic light scattering was used to determine the size distribution of the liposome preparation. A small aliquot of sample, prepared without carboxyfluorescein, was diluted to 2 ml with distilled water and light scattering was recorded at an angle of 90° in a thermally

jacketed sample chamber maintained at 21.3°C. Measurements were made at three sample times using a helium-neon laser (wavelength 632.8 nm), a quantum photometer, and a 64-channel autocorrelator (Langley-Ford model 1096). Analysis of the resulting autocorrelation functions was carried out using the method of cumulants.

Results

Vesicle morphology and size distribution

Freeze-fracture electron microscopy and light scattering studies revealed that the liposomes prepared with a molar ratio of 7 : 2 : 1 (phosphatidylcholine/cholesterol/stearylamine) were composed almost exclusively of unilamellar vesicles (some oligo- and multilamellar vesicles were observed). These ranged in size from 0.4 to 4.4 μm in diameter (Fig. 1). The average vesicle diameter was 1.71 μm. While the other liposome compositions would produce vesicles of different sizes, they should remain essentially unilamellar by virtue of the preparation method used [18].

Tear-induced release of liposome-entrapped carboxyfluorescein

Carboxyfluorescein-release experiments revealed that, in the presence of rabbit tear fluid, liposomes lacking cholesterol rapidly released most of the entrapped dye within 1 h (Fig. 2A). This tear-induced leakage, however, could be reduced by incorporating cholesterol in the liposomal bilayers in proportions ranging from 20 to 45 mol% (Fig. 2B and C).

A comparison of the effects of cholesterol content on tear-induced leakiness is illustrated in Fig. 3. In vesicles prepared with no cholesterol (9 : 0 : 1), 80% more dye was released within the first hour, relative to the controls, with a greatly reduced, but continued, rate of release thereafter. Liposomes prepared with equimolar amounts of phosphatidylcholine and cholesterol (4.5 : 4.5 : 1) were still susceptible to tear-induced leakiness; however, the average amount of dye released was reduced to less than 19% of controls during the 5 h course of the experiment. At the intermediate composition (7 : 2 : 1), tear-induced dye release was still pronounced, but gradual, with 41.5% more dye being released at 1 h. This release increased to 65.7% after 5 h.

Half-times for carboxyfluorescein leakage

The half-times for carboxyfluorescein release, calculated using first-order kinetics, are shown in

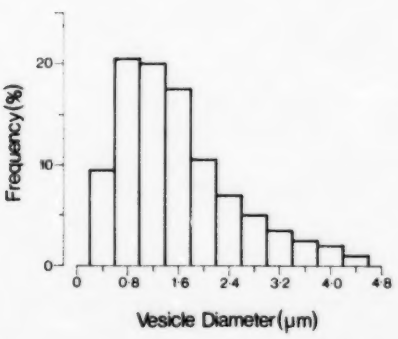

Fig. 1. Freeze-fracture micrograph and size distribution histogram for reverse evaporation vesicles. The liposomes were prepared using egg phosphatidylcholine, cholesterol, and stearylamine (7 : 2 : 1, molar ratio) and were quenched from room temperature for freeze-fracturing. Quasi-elastic light scattering was used for vesicle sizing as described in Materials and Methods.

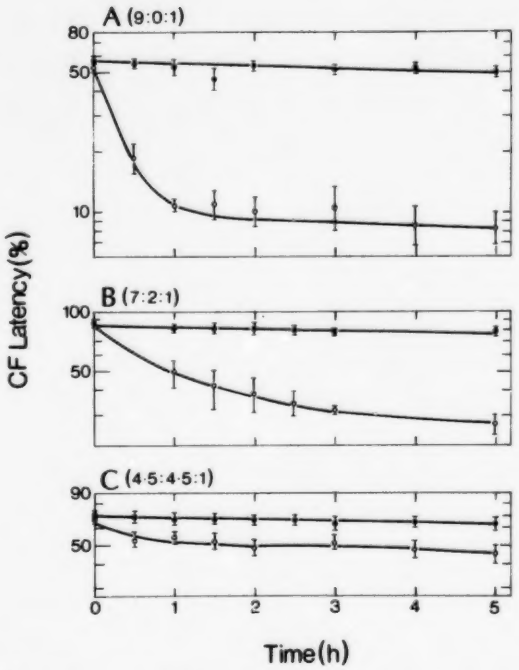

Fig. 2. Effect of rabbit tear fluid on carboxyfluorescein release from unilamellar liposomes. Liposomes were prepared from egg phosphatidylcholine, cholesterol, and stearylamine in molar ratios of 9:0:1 (A), 7:2:1 (B) and 4.5:4.5:1 (C). The vesicles were incubated at 37°C in an equal volume of either buffer (●) or tear fluid (○). Latency values are means ± S.E. from at least three independent experiments.

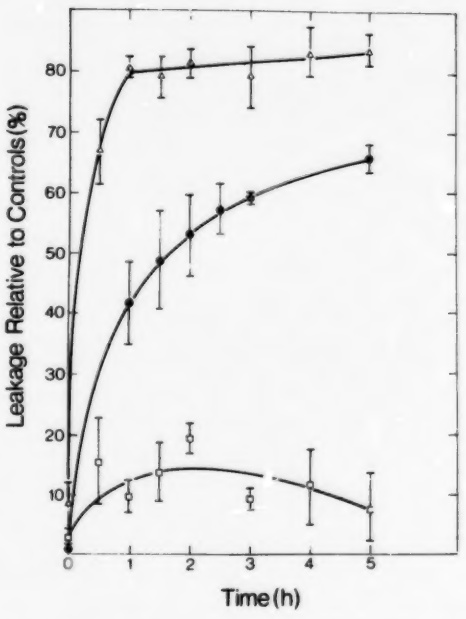

Fig. 3. Effect of liposomal cholesterol content on relative tear-induced dye release. Liposomes were prepared with molar ratios of 9:0:1 (△), 7:2:1 (●), and 4.5:4.5:1 (□) using phosphatidylcholine, cholesterol and stearylamine. For each experiment, tear-induced carboxyfluorescein release was calculated relative to identical liposomes incubated in buffer as indicated in Materials and Methods. Data represent means ± S.E. from at least three independent experiments.

TABLE I

HALF-TIMES ($T_{1/2}$) FOR CARBOXYFLUORESCEIN RELEASE FROM CHOLESTEROL-RICH AND CHOLESTEROL-FREE LIPOSOMES IN THE PRESENCE OF INCREASING CONCENTRATIONS OF TEAR FLUID AT 20°C

The procedure for the determination of carboxyfluorescein release half-times is described in Materials and Methods. Number in brackets indicates number of experiments. S.E. are reported where $n = 3$. n.d., not determined.

% Tear fluid	$T_{1/2}$ (h)	
	zero cholesterol	45 mol% cholesterol
0	6.96 ± 1.03 (3)	60.62 ± 9.02 (3)
10	n.d.	9.40, 7.54
25	1.65, 2.02	10.58, 7.22
50	0.65, 0.79	7.57, 4.93
100	0.43 ± 0.05 (3)	6.59 ± 1.59 (3)

Fig. 4 and are approximately one order of magnitude less in the presence of tear fluid. Preparations incubated only in Hepes buffer showed an expected increase in half-times rising from 7.0 to 60.5 h as the proportion of cholesterol was increased from 0 to 45 mol%. The ability of cholesterol to protect the vesicles against tear-induced release is reflected by the roughly parallel increase in half-times observed in the presence of tear fluid. Using the 9:0:1 vesicle composition, the half-time for carboxyfluorescein release was 0.43 h. This increased to 6.6 h at the highest cholesterol content used.

The effect of tear concentration on the half-times for dye release was also determined and the results are presented in Table I. Only two liposome compositions were utilized; 9:0:1 and

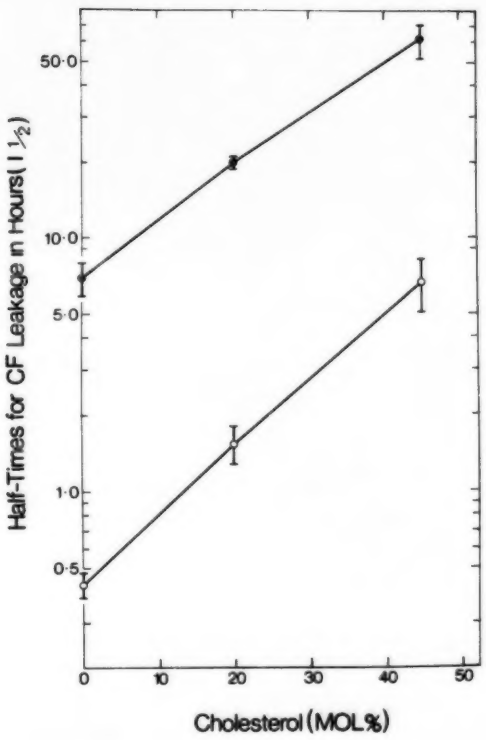

Fig. 4. Effect of liposomal cholesterol content on the half-times for carboxyfluorescein leakage from liposomes incubated in buffer (●) or rabbit tear fluid (○). $T_{1/2}$ was calculated as indicated in Materials and Methods. Data represent means ± S.E. from at least three independent experiments.

4.5 : 4.5 : 1. Using the cholesterol-rich vesicles, the greatest change in the half-time occurred with the lowest concentration of tear fluid, namely 10%. The half-time in this case dropped dramatically from 60.6 h to 8.5 h. Thereafter, there was relatively little further change as the tear concentration was increased through to 100%. Interestingly, although roughly the same magnitude of change was observed with cholesterol-free liposomes, a more gradual decrease in half-times was observed.

Discussion

While the ability of serum to increase liposome permeability has been reported [2,4,19], little is known about the effect of tear fluid on liposomes. The results of this study clearly demonstrate that rabbit tears can compromise liposomal integrity as measured by the release of an entrapped marker. Large unilamellar liposomes were prepared from egg phosphatidylcholine, stearylamine and varying amounts of cholesterol. Their stability was monitored by following the release of the water-soluble, self-quenching fluorescent dye, carboxyfluorescein. Using this method, the half-times for dye release, at all cholesterol levels examined, were approx. 10-times less in the presence of tear fluid then in the presence of Hepes buffer alone.

In the presence of rabbit tear fluid, positively charged cholesterol-free phosphatidylcholine liposomes rapidly lost most of their entrapped dye within 1 h. Both the rate (Fig. 2) and the extent of this loss (Fig. 3) could be reduced by incorporating increasing amounts of cholesterol in the liposomal membranes. Cholesterol is known to have a condensing effect on liquid-crystalline phosphatidylcholine bilayers and this results in a decrease in permeability [20,21]. The reduction in tear-induced leakage brought about by the inclusion of cholesterol in the vesicle bilayers suggests that the responsible tear components may require access to the region of the phospholipid fatty acid chains.

The membrane-destabilizing effect of tears resembles that of serum where increased vesicle permeability is largely the result of bilayer destabilization by high density lipoproteins (HDL) [19,22,23]. The observed loss of vesicle contents has been attributed to either the formation of pores (without total vesicle disruption) [24] or to total vesicle destruction [25]. Leakage can be reduced by the inclusion of cholesterol and by other manipulations that serve to tighten the molecular packing of the bilayer [3,4].

To our knowledge, there are no reports of HDL in tear fluid. However, numerous proteins have been reported and these include various immunoglobins, albumin, lysozyme, and several other enzymes [26]. Whereas the molecular weights of human HDL_2 and HDL_3, both of which interact with liposomes [27], are 320000 and 175000 respectively [28], molecular species over 150000 in size are only occasionally found in rabbit tears [29]. Therefore, it seems rather unlikely that the liposome-destabilizing factors of serum and tears are the same. The tear fluid factor responsible for

the observed increase in permeability remains to be determined.

Whatever the responsible factor, it apparently is present in excess because tear fluid, diluted to as low as 10% in concentration, still can cause a near-maximal reduction in the half-time for dye release from cholesterol-rich liposomes. A similar release pattern was observed by Allen and Cleland [2] with serum. The tear-dilution experiment indicates an apparent resistance of the cholesterol-free liposomes to the tear factors. If this resistance is the result of some saturation phenomenon or some other limiting factor, normal lacrimal flow in vivo would ensure replenishment of the active tear components.

Some success has been achieved in the utilization of liposomes as ophthalmic drug delivery devices. For example, improved uptake of triamcinolone acetonide [14], penicillin G and indoxole [12], and inulin [15] have been reported. The relatively few studies which have been undertaken indicate the importance of several factors, including liposomal surface charge [12,14,30], size [12] and the affinity of the drug for the liposome [12,15,30]. Stratford and co-workers [15] have determined that epinephrine, unlike the larger (but also water-soluble) inulin molecule, is rapidly released from its liposomal carrier when instilled in the rabbit eye. Liposomal delivery of these compounds results in a substantially reduced uptake of epinephrine, while inulin uptake is increased 10-times. Citing unpublished data, the above authors reported that liposomes, exposed to tear proteins, rapidly released entrapped epinephrine but were refractory to the release of inulin. These results suggest that total liposome lysis need not occur.

Clearly, the stability of liposomes in tear fluid is a significant factor in their application as delivery devices for ocular drug delivery. This must also apply to lipophilic and amphipathic drugs which have been proposed to gain entry to ocular tissues by direct membrane-to-membrane transfer [12]. Liposome disruption, such as that suggested to occur in the presence of HDL [25], would preclude the necessary adsorption and close apposition of liposomal and corneal surfaces. We have demonstrated in this study that tears increase liposome permeability to a water-soluble dye and that cholesterol can reduce both the rate and the extent of tear-induced release. Whether the observed release is the result of increased permeability only, or whether vesicle destruction also occurs, remains to be established. Further studies must be undertaken to establish the factors and conditions controlling drug release for the effective application of liposomes as drug carriers for topical ophthalmic delivery.

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